

Day 1: Plug Equilibration In Digestion Buffer

1. **Remove needed plugs from 0.5 M EDTA (storage buffer)** and put one per 1.5-mL Ependorf tube filled with 1 mL water.
2. **TE washstep 1.** After 15 min., remove water as completely as possible with a pasteur pipet. Add 1 mL of TE containing 0.1 mM EDTA (0.01 M Tris-HCl and 0.1 mM EDTA, pH 7.4). Rotate 360 degrees for 1-2 h in cold room or at room temperature.
3. **TE washstep 2.** Remove TE as completely as possible with a pasteur pipet. Add 1 mL more of TE. Rotate for 1-2 h in cold room or at room temperature.
4. **Equilibration.** Remove TE as completely as possible with a pasteur pipet. Add 1 mL of the digestion buffer. Rotate for 2-3 h at room temperature or cold room. Leave overnight in the refrigerator. Or overnight in cold room. *Do not rotate over the weekend because then the plug might be damaged.*

Day 2: Digestion

1. **Prepare the Master Mixes** with buffer and enzyme and put on ice. Keep enzymes in refrigerator just until use.
2. **Remove plugs from the tubes** with a small spatula and split the plugs in half with a scalpel down the middle transversely so that you have two almost square halves. Put one half in the new tube and put back one half in the original tube with equilibration buffer.
3. Without delay, **add 150 uL of the Master Mix** of digestion buffer, spermidine, and enzyme(s) EcoRI, HindIII, BlnI, and/or XapI.
4. Do EcoRI/HindIII, EcoRI/BlnI, EcoRI, XapI, HindIII and NotI digestions for 6 h or overnight at 37 C

**Day 3: Pulsed Field Electrophoresis**

1. **Prepare 5 L of running buffer,** 0.5 X TBE with 75 uL of 10 mg/mL EtBr. Remove old running buffer from the gel apparatus and replace with about 2,5 L of the freshly prepared buffer.
2. **Prepare a 0.88% agarose gel** (2.65g in 300 mL 0.5x TBE) for a 20 x 20 cm gel using agarose that is suitable for PFGE (MP agarose Roche). Boil solution to dissolve agarose, cool down until 70 C and add 4.5 µl EtBr (per 300 mL).
3. **Gel preparation**
 - a. **Pour the gel** gradually so you do not get bubbles. Let the agarose harden for at least 45 min at room temperature or pour in the cold room and let harden for at least 15 min.
 - b. Place a little water on the gel in front and in back of the comb. Keeping the gel in place carefully and gradually (without stopping) **remove comb**. Immediately put water in the wells. Immediately wash the comb and erlenmeyer flask so that no gel remains in them.
4. **Sample loading.** Have two yellow tips, one to push the gel slice and the other to mark the next lane for sample addition by leaving it on the gel pointing down to the lane. Slide a black laminated piece of film under the top of the gel to increase the contrast so that you can see the wells and distinguish full from empty. For each ½ gel plug, position it vertically on end of the spatula. Avoid touching the gel. Position a little end of the spatula in the well push the plug gently (not tearing it) with the end of a yellow pipet tip or with your gloved finger, so that it is below the top of the gel. *(You could add 2 uL of 5x bromophenol blue running dye to a well just before adding a plug).*
 Add the marker last: λ HindIII digest in bromphenol blue dye and concatemered λ in agarose (Midrange PFGE Mark 50 ug/mL, NEB). The concatamer marker should be placed as the first and last sample with at least one lane empty between them and the ends of the gel.

Rinse the spatula and tip used for handling concatamer plugs. Cut the thinnest possible slice just before you need to use it and then cut only 1/2 of that. (*Unlike liquid samples, gel samples can electrophorese properly even if there is leakage but if the λ leaks, do not hybridize the blot with a mixture of λ probe and p13E11 probe but instead, use the p13E11 probe first and then strip the blot for the marker probe hybridization.*)

5. **Put your gel in the PFGE chamber.** Add more electrophoresis buffer so that it is about 1 cm on top of your gel.

The settings for electrophoresis recommended for the BioRad Chef II are for EcoRI/HindIII, EcoRI/BlnI, or HindIII, 1 sec as start and 20 sec as stop time at 21.5 C (2 identical cycles of 10 hours). The only differences for the NotI digests are 3 as start and to 35 sec as stop for 46 hours. Lower temperature makes run slower. 18 or 20 C on BioRad should be OK.

Day 4. Blotting.

Wear gloves whenever you touch membrane and do not get any dirt on it. Wear safety glasses and a lab coat when working with the NaOH/NaCl solution.

1. **Prepare the blotting solution** (NaOH/NaCl, 80g NaOH, 175g NaCl in 5L water).
2. **Prepare the blotting towels** (40 X 60 cm cellulose sheets ('celstof') by folding them so that they are the size of the gel and each individual towel is not too thick. Need about 12 cm layer total.
3. **Cut three pieces of special thick blotting paper** (GB003 gel blotting paper) so that they are the size of the gel.
4. **Cut the two pieces of the paper bridge** (GB003 gel blotting paper); keep them together to wet in NaOH/NaCl and add to the transfer apparatus as one unit.
5. **Cut the membrane** (Hybond; XL; charged nylon membrane; RPN203S 20cm x 3m in a roll) to the size of the gel. Label the bottom of the membrane with a waterproof black pen.
6. **Preparation of blot procedure.** (During the last 15-min incubation of the gel. Pre-wet membrane in water and put it with written side down. The membrane is then put in the NaOH/NaCl transfer solution for 5 min.
7. **Get gel from PFGE apparatus.** Turn off cooler for PFGE apparatus when the electrophoresis turns off according to preset time (or up to 4 h later). Gently lift the gel from one side supporting it with a cradling hand.
8. **Take a picture of the gel under UV** light. Irradiate the gel with UV (transilluminator) for about 30 sec to nick the DNA. Alternatively, irradiate the gel with a Stratalink (autocrosslink).
9. **Denaturation.** Cover with other transfer plate and rotate the gel 180 degrees so that the bottom of the gel is now on the top. Put the gel in a small basin for the denaturation step. Pour the NaOH/NaCl to cover it. Move the basin back and forth gently if you see bubbles between the gel and the plate but bubbles underneath the plate are OK. Gently shake the gel in the NaOH/NaCl for 15 min. Refresh the NaOH/NaCl and shake for another 15 min. Be prepared for putting gel on blotting setup so that you start it by 15 min after that (total time from initiation of shaking = 30 min).
10. **Gel placement.**
 - a. Build up the bridge: pre-wet the double-thickness bridge in NaOH/NaCl and gently, but quickly, put it on the plastic plate on top of the plate atop the small tray, immediately gently lower the flaps of the bridge into the NaOH/NaCl.
 - b. Immediately after completion of the last 15 min NaOH/NaCl soak of the gel, slide the gel onto the bridge; the bottom of the gel should be facing up because you turned the gel upside down after the transilluminator exposure.
 - c. Gently lower the membrane that has pre-soaked in NaOH/NaCl on the square part of the bridge aligning it well over this part of the bridge.

- d. Prewet first blotting paper sheet in NaOH/NaCl and slowly lower exactly in place over the membrane from one end to another. Lift the edge of the membrane and put first 4 strips of plastic to make a picture frame between the membrane and the gel. With a cut-off 10-mL pipet starting from the middle of the gel region, roll out air bubbles to both ends.
 - e. Pre-wet the second blotting sheet and lower it exactly into place. Roll out air bubbles as above.
 - f. Put the third and last piece of blotting paper on dry. Roll out air bubbles and keep rolling until it is completely wet.
 - g. Put the first of the folded towels on top and then put on the rest up to ~12 cm height of towel stack.
11. **Add more NaOH/NaCl** until reservoir is filled completely.
 12. **Cover paper towel with a plastic plate and leave overnight.**

Day 4. Storing blot or proceeding with hybridization the same day.

1. **Membrane release.**
 - a. After blotting **transfer membrane to SSC/Tris neutralizing solution** for 10 min
 - b. **Dry for at least 30 min between clean paper towels**, prior to UV crosslinking. Store at room temperature, before prehybridization and hybridization

Day 4 or after Storing Blot: Prehybridization and Hybridization

1. **Crosslink DNA to membrane** with Stratagene 1200 using the autocrosslink setting.
2. **Cut the membrane** in two pieces, the 19 x 19 cm membrane is cut in half along the length, approximately in the middle lane of the gel, parallel to the lanes.
3. **(Pre-)hybridization** is done for at least 1 hour in 90 mL of hybridization buffer for big trays, 25 mL. for oven tubes.

Hybridization conditions for following probes:

p13E11, B31, 4qA, 4qB (65°C)	D4Z4 (62°C)
125 mM NaHPO ₄ , pH 7.2	125 mM NaHPO ₄ , pH 7.2
10% PEG 6000	50% formamide
0.25 M NaCl	0.25 M NaCl
1 mM EDTA	7% SDS
7% SDS	
1% 10 mg/mL Fish sperm*	1% 10 mg/mL Fish sperm*

* add 1mL/100 mL hy buffer after heating to 95 degree for > 10 min

4. **Probe preparation according to megaprime protocol (Amersham).**
 - a. **Preparation lambda DNA probe** (can be stored for 2 months, just use more probe as radioactivity decreases)
 - 4 uL of lambda DNA (25 ng) from the control from the Megaprime kit,
 - 2 uL of primer,
 - 7.5 uL H₂O,
 denature at 95 C for 5 min; cool to RT 5 min, then centrifuge shortly.
 Add to denatured DNA sample:
 - 1 uL Klenow,
 - 4 uL 5X buffer,

- 1.5 uL ³²PdCTP (6.5 uL).
- add TE with stop dye until to 500 uL.

b. Preparation specific probe

- x uL DNA stock (20-40 ng) diluted to 11.5 uL with water
 - 2 uL primer
- denature at 95 C for 5 min; cool to RT 5 min, then centrifuge shortly.
Add to each denatured DNA sample:
- 1 uL Klenow,
 - 4 uL 5X buffer,
 - 1.5 uL ³²PdCTP (6.5 uL).

Incubate at 37 C for 20 min. Add 80 uL TE with stop dye / reaction.

Mix 2 uL of lambda probe to 100 µl specific probe. Denature 7 min at 95C, place on ice.

Note: we do not perform a Sephadex- separation of labeled probe from free dNTPs

6. **Hybridization step.** Make the hybridization solution by adding the labeled probe to the pre-hybridization solution: lift blots from solution, add probe and 1 by 1 place back blots. Hybridize overnight.
7. **Wash 3 times at 65°C for 8-10 min:**
 - p13E-11 probe : 3x 2xSSC, 0.1% SDS.
 - B31 probe : 1x 1xSSC. 0.1% SDS; 2x 0.3x SSC with 0.1% SDS.
 - 4qA probe : 1x 2xSSC. 0.1% SDS; 2x 1xSSC with 0.1% SDS
 - 4qB probe : 1x 1x SSC. 0.1% SDS; 2x 0.3x SSC with 0.1% SDS
 - D4Z4 probe : 3x 0.1x SSC with 0.1% SDS
8. **Place in RT 2x SSC buffer and seal blots**
9. **Imaging the blot.** Expose to phosphor imager for 1 to 2 days.
10. **(optional) Strip blot to remove old probe signal** by adding boiling 0.1x SSC, 0.1% SDS solution (microwave until it just starts to be at a full boil) to membrane. Then leave about 10 minutes and then pour off.